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TITLE: Regulatory T Cell-Enriching Microparticles for Promoting Vascularized Composite Allotransplant Survival

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14. ABSTRACT

The purpose of this work is to investigate the ability of engineered biomimetic drug delivery systems to prevent rejection and promote immunological tolerance in the context of composite tissue allotransplantation (CTA). For this reporting period our goals were to fabricate and characterize microparticles to be used in animal surgeries, achieve IACUC/ACURO approval for animal work and start the first phase of rodent hindlimb transplants. Particles containing IL-2,TGFβ, and rapamycin were fabricated and this triple cocktail, along with all pairwise iterations of the three components were tested for their ability to prevent hindlimb rejection (via 2 subcutaneous injections). Data at present seems to suggest that the combination of all three factors yields the best results, however some groups are still in the follow up period and accordingly, no statistical significance can be claimed until the follow up period is completed (this will happen within the next month).

15. SUBJECT TERMS
Microparticle, Regulatory T Cell, CTA, Transplant, Biomimetic

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Introduction

Millions of Americans have sustained unsalvageable tissue loss secondary to trauma, sepsis/disease, cancer, and congenital defects. In most cases, current reconstructive strategies are sub-optimal or fail to provide optimal results in terms of aesthetic or functional outcomes. For these patients, composite tissue allotransplantation (CTA), encompassing transplantation of hands and face is becoming an increasingly explored strategy with nearly 200 different types of clinical transplants performed over the past decade. Despite promising results and advances in microsurgical approaches, graft rejection and the deleterious effects of lifelong, high-dose, multi-drug immunosuppression have prevented the broader clinical application of CTA. This project will investigate the potential of using biomimetic microparticles to promote long-term CTA survival in the absence of systemic immunosuppression via the in situ recruitment and expansion of a patient's own suppressive regulatory T cells. These particles, referred to as Expansion MP (IL2, TGF, and Rapamycin microparticles) and Recruitment MP (CCL22 loaded microparticles) will be tested in both rodent and swine models of CTA. Finally, we will investigate immunobiological mechanisms behind any of the observed effects these particles have on allograft survival.

Key Words

CTA	Composite Tissue Allotransplantation
VCA	Vascularized Composite Allotransplantation
DC	Dendritic Cell
Treg	Regulatory T cell
FoxP3	Forkhead Box P3
TGF-β	Transforming Growth Factor Beta
IL-2	Interleukin 2
IL-6	Interleukin 6
IFN-g	Interferon Gamma
Rapa	Rapamycin
CCL22	Chemokine Ligand 22
CCR4	Chemokine Receptor 4
MP	Microparticle
GMP	Good Manufacturing Practices
PLGA	Poly (lactic-co-glycolic) acid
ALS	Antilymphocyte Serum
ELISA	Enzyme Linked Immunosorben Assay
PCR	Polymerase Chain Reaction
IHC	Immunohistochemistry
MHC	Major Histocompatibility Complex

Accomplishments

What were the major goals of the project?

The following goals/tasks have been completed and/or are in progress.

1. Fabrication of Recruitment and Expansion MPs that release factors as described in the project narrative (Months 1-6): This goal has been completed.
    a. Obtain design parameters from Little Lab predictive model for CCL22, IL-2, TGF-β, and Rapamycin

    b. Fabricate MP formulations using design parameters dictated by our predicted model
    c. Conduct *in vitro* release studies to verify our *in silico* predicted release of CCL22, T TGF-β and Rapamycin over a 30 day period
2. Rat IACUC and ACURO approval (Months 1-6): This goal has been completed.
    a. IACUC protocol write up and approval
    b. ACURO approval following University of Pittsburgh IACUC approval
3. Rat CTA surgery (Months 7-24): This goal is in progress and is ~40% complete and will be completed by the end of year two.
    a. Using microsurgical techniques transplant hind-limbs from Brown Norway rats to Lewis rats
    b. In appropriate groups initiate induction therapy (ALS) on days -4 and +1 and continue maintenance therapy (FK506 0.5mg/kg) for 21 days after transplantation
    c. Inject Recruitment and Expansion MP formulations subcutaneously into the transplanted hind-limb on days 0 and +21
4. Daily monitoring of CTA (Months 6-24): This goal is in progress and is ~40% complete and will be completed by the end of year two.
    a. Monitor transplanted CTA daily for rejection using the following scale Grade 0 (no rejection), Grade I (edema), Grade II (erythema), Grade III (epidermolysis) and Grade IV (necrosis and mummification)
    b. Grafts surviving for greater than 100 days will be considered long term survivors

The following goals/tasks have not begun yet and will take place in years two and three.

5. Immunohistochemistry (Months 24-30)
6. Analyze cytokine gene expression in CTA grafts via PCR (Months 24-30)
7. Demonstrate that our biomimetic MP therapies promote both in vitro and in vivo donor specific tolerance (Months 15-29)
8. Further Biological Analysis (Months 19-33)
9. Pre-IND Meeting with FDA (Month 16)
10. Fabrication of Recruitment and Expansion MP Therapies for Swine CTA Model (Months 13-33)
11. IACUC and ACURO Approval for Swine Surgeries (Months 20-26)
12. Conduct swine gracilis myocutaneous free flap allotransplantation with Recruitment MP and Expansion MP Treatment (Months 14-20)
13. Daily monitoring of CTA grafts (Months 25-35)
14. Biological Analysis of Tissue from Swine CTA Transplants (Months 29-34)

What was accomplished under these goals?

Recruitment MP Fabrication – Key Results and Methodology

Microparticles (MP) containing CCL22 were prepared as follows. Briefly, recombinant CCL22 was mixed with 200 mg of Poly (lactic-co-glycolic) acid dissolved in dichloromethane. This mixture was sonicated (to form the first emulsion of water-in-oil) before being poured into a PVA solution being homogenized (forming the second emulsion). Following homogenization, the solution was mixed with PVA and the dichloromethane was allowed to evaporate. After 3 hr, the freshly formed MP were centrifuged and washed. The MP were then re-suspended in water, frozen on dry ice and lyophilized.

The surface morphology of Recruitment MP was characterized using a scanning electron microscope (Figure 1A) and Recruitment MP size distribution measured using volume impedance measurements on a Beckman Coulter Counter (Figure 1B).
In vitro release characteristics were measured by suspending ~7mg of Recruitment MP in 1ml phosphate buffered saline (PBS) on an end-to-end rotator at 37°C. At different time intervals, the suspensions were centrifuged, the supernatant collected, and the Recruitment MP re-suspended in 1ml PBS. The amount of CCL22 in the supernatant was determined by ELISA (Figure 1C).

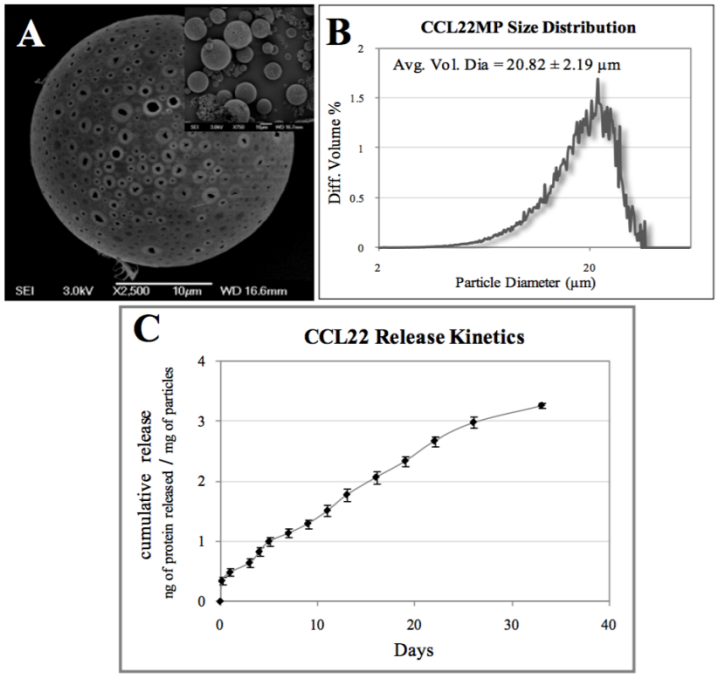

Figure 1: A – scanning electron micrograph of an intact CCL22MP showing its external porous structure. Inset is zoomed-out detail of main image. B - representative volume-averaged size distribution of CCL22MP; average volume diameter measurements ± SEM are based on n=6 particle sets. **C** – release kinetics of CCL22MP as measured in PBS; errors bars represent cumulative SEM based on n=3 experiments.

Sustained release of CCL22 was achieved by loading the chemokine into degradable poly (lactic- co-glycolic) acid-based microparticles (CCL22MP). Scanning electron micrographs of intact MP indicate that they are spherical and slightly porous (Figure 1a). The surface of Recruitment MP was specifically formulated to be porous, to allow continuous release (without periods of lag) of chemokine (Figure 1b), as guided by new mechanistic descriptions of how controlled release of proteins occurs in such systems. Further, the particles were designed to be large enough to avoid uptake by phagocytic cells and to prohibit their movement across vascular endothelium, with consequent immobilization at the site of injection (Figure 1c).

Expansion MP Fabrication – Key Results and Methodology
IL-2 and TGF-β microparticles (IL2MP and TGFβMP, respectively) were prepared using the double emulsion-evaporation technique, as described above with Recruitment MP. Rapamycin microparticles were fabricated as follows. Rapamycin dissolved in DMSO was mixed with dichloromethane containing PLGA. This solution was mixed with PVA under homogenization creating the microparticle emulsion. The resulting emulsion was then added to PVA and left for 3 hours allowing the dichloromethane to evaporate. Subsequently, the microparticles were centrifuged washed, and lyophilized.

Release assays were conducted by incubating a suspension of particles; (i) 10 mg in 1 ml of media for IL2MP and TGFβMP microparticles, and (ii) 10 mg in 1 ml of PBS (containing 0.2% Tween-80) for RapaMP, on a roto-shaker at 37 C. At regular time intervals, particle suspensions were centrifuged, the supernatant removed, and the particles re-suspended in 1 ml of appropriate solution. The amount of cytokines in the supernatant was measured using a cytokine-specific ELISA, and the amount of Rapa was measured using spectrophotometry (Figure 2, bottom panel). IL2MP, TGFβMP, and RapaMP were all prepared under similar conditions, using the same polymer (RG502H, viscosity 0.16-0.24 dl/g). Scanning electron micrographs (Figure 2, top panel) show that individual particles are spherical and confirm the volume average size distributions (IL2MP = 25.5 ± 7.5 μm; TGFβMP = 16.7 ± 6.3 μm; rapaMP = 16.7 ± 6.4 μm). Additionally, the images show that IL2MP have slightly porous exterior surfaces. These particles were specifically formulated to be porous (by altering osmotic pressures between the inner emulsion and the outside aqueous phase during microparticle preparation) so that a high initial burst followed by continuous release could be obtained (Figure 2, bottom panel). Further, we observe a linear release of TGF-β following a ~2 week lag phase, and a continuous release from RapaMP (Figure 2, bottom panel).

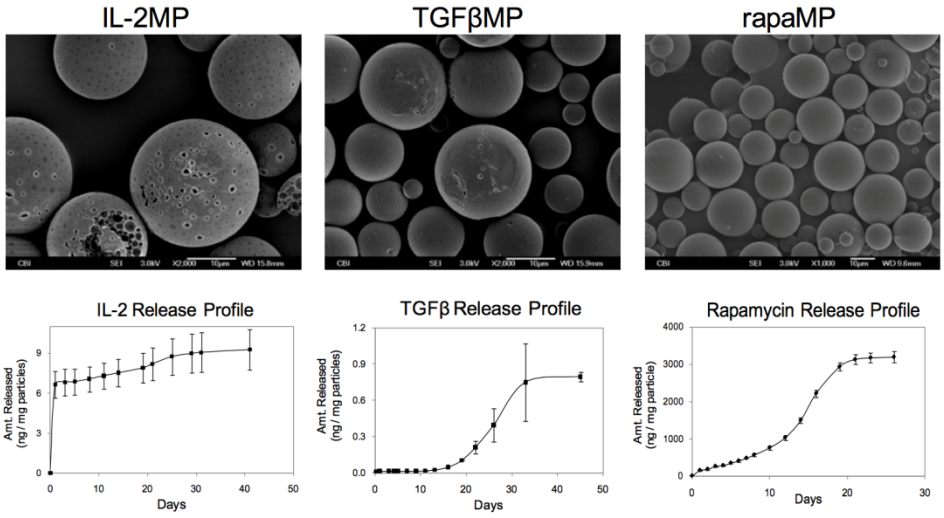

Figure 2: Scanning electron micrographs (top panel) and in vitro release profiles (bottom panel) of IL-2, TGFβ and Rapamycin.

Rat Hind Limb CTA with Expansion MP Therapy Methodology

While our MP therapies are aimed at long term survival, all animals in all groups will receive the same baseline CTA immunosuppression protocol developed at the University of Pittsburgh's CTA laboratory. This protocol consists of 21 days of systemic FK-506 (IP injections) at a concentration of 0.5mg/kg as well as two 0.5cc IP injections of ALS induction therapy on days -4 and +1 relative to the transplant (Figure 3). This baseline immunosuppression protocol is used to provide the necessary support for animals in the acute postoperative

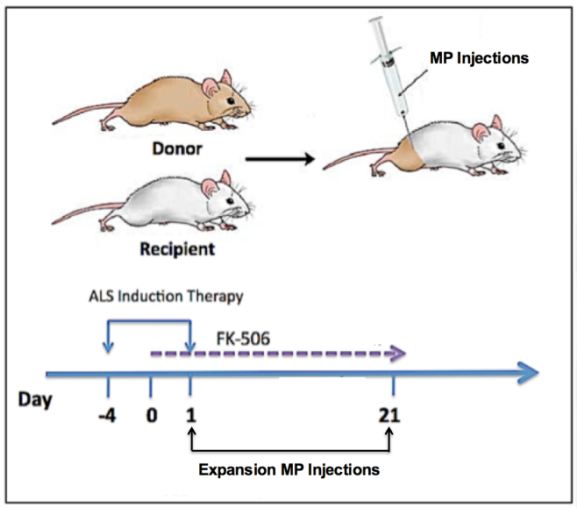

Figure 3: Therapeutic Timeline for Hindlimb Transplants

period and will (most importantly) demonstrate that our technologies can be used in tandem with conventional immunosuppression.

Surgical Follow Up - Methodology
All controlled release treatments will be injected subcutaneously (at a concentration 10mg/ml) using a 25G insulin syringe at the time of transplantation and again on postoperative day 21. To assess rejection, animals were monitored daily and scored for rejection (appearance grading) based on physical examination. Animals were given a daily score using the following scale: Grade 0 (no rejection), Grade I (edema), Grade II (erythema and edema), Grade III (epidermolysis) and Grade IV (necrosis and "mummification"). Grafts were considered rejected when displaying signs of progressive Grade III rejection.

Surgical Follow Up – Key Results
Expansion MP therapies were tested for this reporting period. Specifically, we looked at the effect Expansion MP to prolong CTA survival in the absence of long term systemic immunosuppression. Because Expansion MP is a triple cocktail it is imperative to test all iterations of the components of Expansion MP to determine the necessary and minimal combination of factors needed to generate immunological tolerance. Thus, the following groups were tested: 1) Expansion MP, 2) IL2MP, 3) TGFMP, 4) RapaMP, 5) IL2MP+TGFMP, 6) IL2MP+RapaMP, 7) TGFMP + RapaMP, 8) Expansion MP injected in the contralateral limb, and 9) no microparticle treatment (FK506/ALS only). The results to date are summarized below in Table 1. Because all groups receive the same baseline immunosuppression protocol for 21 days, animals under the cover of systemic FK506 should not demonstrate signs of rejection until the FK506 is discontinued at day 21. As such animals in Table 1 still under the cover of FK506

Treatment Received	N	Onset of Rejection (Postoperative Day)
Expansion MP	13	>100 (n=12), 44
IL2MP	5	32, 35, 38, 40, 39
TGFMP	6	40, 38, 38, 35, 80
RapaMP	5	38, 37, 35, >100, >100
IL2MP+TGFMP	6	N/A (n=6)
IL2MP+RapaMP	6	38, 42, 44, 44, >100, >100
TGFMP + RapaMP	6	41, 44, >100, >100, N/A (n=2)
Contralateral	6	N/A (n=6)
No Treatment	6	37, 38, 38, 39, 40, 40

Table 1: Summary of Hindlimb Survival Following Microparticle Treatment

as of the writing of this report are designated as "N/A". Our results to date appear to suggest that the triple cocktail of IL2MP, TGFMP and RapaMP is the more effective than any of the pairwise controls, however we are still monitoring multiple animals in these control groups that are still receiving FK506 and cannot make any conclusions until the follow up period is completed for all groups.

What opportunities for training and professional development has the project provided?
Nothing to Report.

How were the results disseminated to communities of interest? Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
During the next reporting period we will continue daily monitoring of the grafts for signs of rejection from the Expansion MP study. Furthermore, animals that have reached the end of the follow up period with no signs of rejection will sacrificed and the relevant tissues harvested and processed. Finally, we will begin testing the Recruitment MP formulations and have animal surgeries planned.

Impact

What was the impact on the development of the principal discipline(s) of the project? Nothing to Report

What was the impact on other disciplines? Nothing to Report

What was the impact on technology transfer? Nothing to Report

What was the impact on society beyond science and technology? Nothing to Report

Changes/Problems

Nothing to Report.

Products

Publications, conference papers, and presentations

1. Fisher, James D, Schweizer, R, Unadkat JV ,Fries, A, Komatsu C, Oksuz S, Solari MG, Davis M, **Gorantla VS**, **Little SR**. Biomimetic Microparticles can Establish Dominant Tolerance in Vascularized Composite Allotransplantation via Endogenous Regulatory T Cell Enrichment. 15th Annual McGowan Institute Retreat, Nemacolin Woodlands, PA March 6-8 2016. (Oral and Poster Presentation)

2. Fisher, James D, Schweizer, R, Unadkat JV ,Fries, A, Komatsu C, Oksuz S, Solari MG, Davis M, **Gorantla VS, Little SR**. Biomimetic Delivery of Regulatory T cell Enriching Factors Establish Dominant Tolerance in Vascularized Composite Allotransplantation. 13th US-Japan Symposium on Drug Delivery, Maui HI, December 16-20 2015. (Oral and Poster Presentation)

Website(s) or other Internet site(s): Nothing to Report

Technologies or techniques: Nothing to Report

Inventions, patent applications, and/or licenses: Nothing to Report

Other Products: Nothing to Report

Participants and Other Collaborating Organizations

What individuals have worked on the project?

Name:	Steven Little
Project Role	PI
Researcher Identifier	0000-0002-7000-3931
Nearest person month worked	1.5
Contribution to Project	Dr. Little is responsible for leading this project. This includes all experimental planning as well as troubleshooting with respect to microparticle formulation and characterization

Name:	Vijay Gorantla
Project Role	Co-I
Researcher Identifier	0000-0003-0686-059X
Nearest person month worked	1.2
Contribution to Project	Dr. Gorantla is responsible for experimental design for this project and trouble shooting especially with respect to animal CTA and immunobiology.

Name:	James Fisher
Project Role	Graduate Student
Researcher Identifier	
Nearest person month worked	12
Contribution to Project	Mr. Fisher is responsible for fabrications of all microparticles used in this project as well as surgical assistance and long term animal follow up.
Funding Support	NIH/NIAID T32 AI 074490, "Interdisciplinary Training in Transplantation Biology"

Name:	Liwei Dong
Project Role	Postdoctoral Fellow
Researcher Identifier	0000-0002-6122-7677
Nearest person month worked	4
Contribution to Project	Dr. Dong is an experienced microsurgeon who completed a portion of the hindlimb transplants during this reporting period

Name:	Ali Mubin
Project Role	Postdoctoral Fellow
Researcher Identifier	
Nearest person month worked	3
Contribution to Project	Dr. Mubin is an experienced microsurgeon who completed a portion of the hindlimb transplants during this reporting period.
Funding Support	Internal/Departmental Funds

Name:	Zhaoxiang Zhang
Project Role	Postdoctoral Fellow
Researcher Identifier	
Nearest person month worked	3
Contribution to Project	Dr. Zhang is an experienced microsurgeon who completed a portion of the hindlimb transplants during this reporting period
Funding Support	Internal/Departmental Funds

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Nothing to Report.

What other organizations were involved as partners? Nothing to Report.